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TITLE: Stroma-Based Prognosticators Incorporating Differences between African and European Americans

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our objective is to identify and validate RNA prognosticators for prostate cancer in European Americans (EA) and African Americans (AA), and port these to DNA methylation and protein-based prognosticators, where possible. The unique aspect of this project is that it uses tumor-adjacent stroma as a source of markers, and uses standard Formalin-fixed Paraffin-embedded (FFPE) tissue, which is a challenge for molecular biology. In the first year we accumulated 70 AA prostatectomy samples, and matched them with 140 EA by Gleason Score, chemical relapse status, and approximate age. We generated both RNA and DNA from 40 AA and 70 EA samples. We can profile the higher quality and higher yield RNA and DNA samples by array card RT-PCR, and bisulfite sequencing, respectively. For the EA samples, we chose the better quality among more than one EA sample mapped to the same AA sample. Exploiting the recent three-fold reduction in the cost of sequencing per read, we developed sequencing methods to extract expression and DNA methylation information from samples in the bottom half of quality and yield, which would otherwise be lost to the study. Many of these samples are among the more important older samples and AA samples.

15. SUBJECT TERMS
Prostate, Stroma, Biomarkers, African American, DNA methylation, RNA, Prognosis.

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1. Introduction…….

Each prostate tumor is heterogeneous, with many DNA and RNA alterations. This obstructs identification of reliable biomarkers in tumors. In contrast, there are relatively few DNA alterations in the tumor-adjacent stroma, which exhibits hundreds of RNA expression changes in response to tumor progression. This abundant tissue is a potential source of prognosticators for prostate cancer progression. The disease is of different epidemiology in African Americans (AA) and European Americans (EA) and they also differ in the expression of many genes in the stroma, which may reflect differences in the pathways to clinical presentation and metastatic potential. We proposed to validate potentially prognostic biomarkers in both AA and EA patients, taking into account that the optimal prognosticator may differ between races. We also proposed to determine DNA methylation differences and protein expression differences because Formalin-fixed paraffin-embedded (FFPE) samples (the established material used in Pathology) are a more reliable source of DNA and protein than of RNA. An accurate prediction of a high risk of relapse following surgery may be the basis for electing immediate adjuvant therapy following surgery. An improved prognosticator for AA patients would immensely benefit this disproportionally highly affected population.

2. Keywords…….

Prostate, Stroma, Prognosis, Biomarkers, African American, DNA methylation, RNA.

3. Accomplishments……..……...

- **What were the major goals of the project?**

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1. Validation of the stroma-based prognostic classifier(s) for both African and European Americans.		
Major Task 1: Obtain and process FFPE tissue for RNA and DNA isolation and identify prognosticators from training set.	months	Status
Subtask 1: Regulatory review and approval processes, including local Institutional Review Board (IRB) and DoD Human Research Protection Office (HRPO).	1-3	100%
Subtask 2: Select appropriate exon-exon spanning primers for qPCR for the 96 genes to be surveyed.	1-6	100%
Subtask 3: Validate primer quality using bulk RNA.	4-6	100%
Subtask 4: Identify the first set of 60 suitable African American (AA) and 60 clinically matched European American (EA) samples with recurrence status and at least five years of follow-up. Training set.	1-6	100%

Subtask 5: Prepare both RNA and DNA from dissected slides. First 60 pairs of cases. Training set.	3-9	50%
Subtask 6: Validate RNA quality using control primer pairs.	6-9	50%
Subtask 7: Perform initial data collection on 96 validated primer pairs and 120 validated RNAs. Training set.	9-12	10%
Subtask 8: Construct prognosticators for recurrence in AA and EA separately, and in AA+EA combined, with and without clinical parameters. Training set.	12-15	To be done
Milestone Achieved: RNA-based prognosticator for recurrence refined based on training set.		
Major Task 2: Perform blinded randomized test.	months	Locations
Subtask 1: Identify the second set of 60 different AA and 60 clinically matched EA samples with recurrence status and at least five years of follow-up. Include a blinding and randomization step. Testing (validation) set.	6-12	20%
Subtask 2: Prepare both RNA and DNA from dissected slides. Second 60 pairs of cases. Testing/validation set.	12-18	To be done
Subtask 3: Validate RNA quality using control primer pairs.	15-18	To be done
Subtask 4: Perform data collection on validated primers and validated RNAs. Testing/validation set.	18-24	To be done
Subtask 5: Assess performance of prognosticators for recurrence in AA and EA separately, and in AA+EA combined, with and without clinical parameters, using the outside statistician. Testing/validation set.	24-27	To be done
Milestone Achieved: Identification of prognostic power of the refined prognosticator in a randomized blinded trial.		
Specific Aim 2: Test for DNA methylation differences near genes that have prognostic RNA expression differences.		
Major Task 1: DNA quality assessment, PCR optimization, sequencing.		
Subtask 1: Determine which regions near each of the 96 genes in Aim 1 (including control genes) show evidence of differential methylation in our reference set of whole genome methylation profiling.	1-6	50%
Subtask 2: Design unique primer pairs to the relevant regions, based on the sequence after bisulfite treatment.	6-12	10%
Subtask 3: Determine which genes in Aim 1 have a prognostic or race-enriched expression profile after training.	12-15	To be done
Subtask 4: Test up to 50 primer pairs on bulk bisulfite treated DNA. Then multiplex and adjust as necessary.	15-18	To be done
Subtask 5: Determine quality of DNAs from samples that were prepared in Aim 1, Major task 1, Subtask 5 using control primer pairs.	18-21	To be done
Subtask 6: Bisulfite treatment of DNAs that pass the above test and test again for bisulfite treatment quality with control primers.	21-24	To be done
Subtask 7: Screen bisulfite treated DNAs that pass the above test using up to 50 primer pairs arranged in ~10 multiplexes. PCR to add index primers.	24-30	To be done

Subtask 8: Pool all PCR reactions, clean, and apply to an Illumina sequencer for 2 x 150 PE sequencing	30	To be done
Subtask 9: Deduplicate, assemble each target region, and determine the frequency and allele distribution of DNA methylation in each case.	33-34	To be done
Milestone Achieved: Determination of DNA methylation differences in genes of known prognostic or race-enriched expression profile.		
Specific Aim 3: Test for congruence of prognostic RNA expression differences with protein expression differences.		
Major Task 1: Obtain and process FFPE tissue for protein expression analysis.	months	Locations
Subtask 1: Determine which of the 96 candidate proteins from Aim 1 shows evidence of expression in the Human Protein Atlas.	1-6	100%
Subtask 2: Identify suitable antibodies for the subset of proteins that pass the filter above.	6-12	100%
Subtask 3: Determine which genes in Aim 1 have a prognostic or race-enriched expression profile after training. (same as Aim 2, Major Task 1, Subtask 3).	12-15	To be done
Subtask 4: Test antibodies on waste sections of TMA for performance. Compare to control antibodies.	15-18	To be done
Subtask 5: Perform IHC on TMAs and scan with up to 10 functional antibodies.	18-33	To be done
Subtask 6: Perform visual data analysis and quantitation.	21-36	To be done
Subtask 7: Assess which proteins have an antibody staining distribution between patients indicative of a correlation with RNA expression, with prognostics, and/or with race.	24-36	To be done
Milestone Achieved: Identification of proteins whose expression is of prognostic value and/or varies between races.		

- **What was accomplished under these goals?**

Aim 1, major task 1, subtask 1 (regulatory review), *2* (primer design), and *3* (validate primer quality), were completed as planned using the methods in the proposal. Other early tasks completed were *Aim 3 subtask 1* (identify genes expressed in the prostate at the protein level) and *subtask 2* (identify suitable antibodies).

Aim 1, major task 1, subtask 4 (identifying pairs of patients) is critical because the quality of the metadata and matching AA and EA patients will strongly influence the results. For this task we diligently accumulated 70 precious AA samples with good follow-up data from our partner at the Medical University of South Carolina. We have added over 150 additional samples to our collection in the first year. **Table 1** shows the overall patient samples we have now accumulated to be able to meet this goal.

Table 1: Update: samples with known relapse status

Gleason Score	Total cases	AA	AA	E	A	German	(EA) Asian	Relapse	Non-Relapse	Recut not yet rescored	Recut and rescored by us
G5	5		3	2				2	3	3	4
G6	178	28	119	23	3	1	4	142	36	40	32
G7	400	83	244	16	26	7	24	207	145	169	69
G8	59	29	30					35	23	23	5
G9	59	14	22		23			48	11	11	2

In *Aim 1, major task 1, Subtask 5*, we prepared both RNA and DNA from the same sample for 40 AA and 70 EA, including samples that could meet the criteria for *Aim 1, major task 2, subtask 1*. We then validated quality in *Aim 1, subtask 6*. The progress, to date, is summarized in **Table 2**. Many samples and many genes produced adequate quality. However, some samples and some primer pairs did not. Note that we use punches into thick recuts of each block, which means we carefully mark pure tumor-adjacent stroma, and take multiple 1 mm punches. This is very different from taking out a big chunk from an FFPE block. Some of the older FFPE samples with long-term follow-up, in particular, do not have an RNA integrity number (RIN) or yield (or both) sufficient for use in multiple PCR reactions on array cards (Aim 1). Similarly, although we can simultaneously obtain hundreds of nanograms of DNA from FFPE punches, this is not always of sufficient quality for massively in parallel bisulfite PCR of the majority of candidate DNA regions (Aim 2).

The older samples are often the most valuable because they generally have the most clinical follow-up data and some are from minority AA. Rather than make any change our objectives, we have converted some of these samples into libraries, and sequenced them. In the case of RNA of low quality and/or yield, we have used the "Access" method of Illumina, to perform sequence-specific **capture** of coding **RNA**. This has allowed us to establish that we have enough complexity remaining across each gene to be able to do expression analysis for these valuable patients with samples of RIN at or below 2.5, often considered poor quality, and using a mass of 100 ng, far less than could be used to examine our genes using array cards. An exciting development is the ability of the HiSeq 4000 to obtain over three times as many reads as the HiSeq2500, at the same price. In processing the first 12 (otherwise useless) samples

through RNA library production, oligo capture, and sequencing (**Table 2**), we obtained tens of millions of mapped deduplicated reads per sample, while using only 5% of a sequencing lane per sample. Most of our precious lower quality samples may be rescued in this way. The RNA libraries give us expression information on all our candidate genes on samples that cannot use array cards and at a price comparable to the approach we are taking with array cards. Thus, our goal of prognosticator validation is not affected.

Table 2. Prostate stroma FFPE samples processed, to date, for Aim 1 and Aim 2

	AA	EA
Patients	40	70
Mean age	59	62
Gleason 6	2	24
G7	34	46
G8	2	0
G9	2	0
R	15	36
NR	20	40
RIN>2.5	21	36
Array card	4	35
RNA library	12	12
Sequenced	4	8
DNA library	5	10
Sequenced*	2	2

*both methyl and unmethyl antibody

means four sequencing reactions

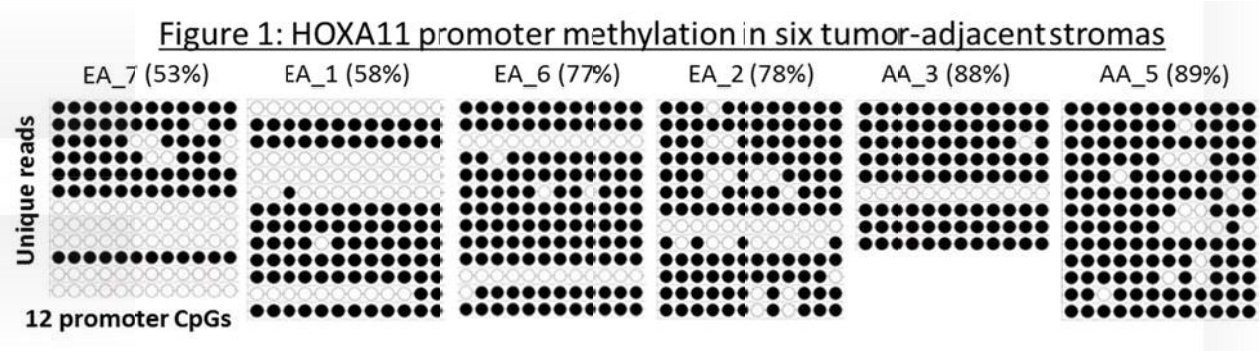
The validation phase we are engaged in, we are using FFPE blocks that are almost *all a lot older than the patient samples that will be used for prognostics*. Thus, there is no concern for fresher FFPE samples being used directly on gene-specific assays, such as array cards.

We are fortunate to be targeting tumor-adjacent stroma. If we were confronting these circumstances with tumor samples, it would be more difficult. We have also been collecting tumor when punching out tumor-adjacent stroma, and archiving it, but pure tumor punches are almost impossible to obtain in many blocks. Indeed, in addition to about 20% of blocks being tumor-negative during our search for tumor-adjacent stroma (which adds to the challenges), an additional 20% have only just enough tumor to be able to identify tumor-adjacent stroma, but not enough pure tumor for collection.

Aim 2, subtask 1 and 2, are focused on DNA methylation in tumor. We identified regions near genes of interest that are differentially methylated in prostate stroma. In some cases we were able to obtain enough DNA to perform bisulfite sequencing. An example for a single gene is presented in **Figure 1** where promoter methylation varies widely among tumor-adjacent prostate stroma samples (each row is an independent clone, each column is a particular CpG and black dots are methylated CpGs).

In the case of DNA of low quality and/or yield, massively in-parallel targeted bisulfite sequencing is out of the question, and we do not want to lose these samples. Making a virtue of necessity, we decided to expand our very small set of genome-wide methylation data using an approach that would work on these samples: the antibody capture methylation profiling described in the grant. We have been able to rescue all four of the samples we have tested (**Table 2**) that were too low yield to be used in massively in-parallel bisulfite sequencing. We were able to obtain tens of millions of deduplicated mapped reads both using methylCpG and unmethyl-CpG antibody and

identify thousands of high confidence differentially methylated locations. With this very encouraging data we will now be able to do full profiling for a subset of the samples and have an improved idea of which locations have the best potential for methylation change associated with recurrence in either AA or EA, or both. All DNA regions within and near all of these genes are assayed for methylation status, including samples we found did not have sufficient yield to be used for large number of bisulfite sequencing PCR reactions.



We are focused on testing our prognosticator by the best available approach, rather than technology development. However, technology for nucleic acid profiling is rapidly changing. For example, a new kit by Illumina has just been released that uses oligo capture for targeted bisulfite sequencing. This strategy focuses on predefined regions of the genome. We will monitor the literature on this approach. For now, our more agnostic approach may yield differentially methylated sites in or near our genes of interest that are not present in the captu ed subset in the commercial kit.

- **What opportunities for training and professional development has the project provided?**

Farah Rahmatpanah, the primary bench researcher, attended two conferences and presented posters (see under “Products”).

This project did not specifically involve training. Nevertheless, in addition to the people providing paid effort and donated effort, listed above, other people have participated in this project without pay, in exchange for training:

- Sepideh Mahadian, MD. P eparing RNA and DNA from FFPE PCa tissues.
- Parsa Rahmatpanah, undergraduate, biology, CSU Fullerton. Preparing DNA and RNA from FFPE PCa tissues.
- Sameer Kapadia, undergraduate, biology, UCLA. Data organization and processing.
- Reza Jahanbakhshi, MD. Scanning tissue microarrays.
- Mehrdokht Boldeji, MD, pathologist. Cell culture, reading H&E slides, Patient data inventories.
- Jenny Song, an undergraduate in biology, UCI. Collating data.

- **How were the results disseminated to communities of interest?**

Outreach include a talk to the MUSC HCC Citizens' Advisory Board in June, 2016 and a talk at a fundraiser for Prostate Cancer Research Foundation in April, 2016

- **What do you plan to do during the next reporting period to accomplish the goals?**

Moving forward with the remaining tasks in Aim 1 is of the highest priority. *Subtask 8 of Aim 1* is the heart of the project and given the low RIN we obtain from many old samples, it is our greatest challenge.

Knowing which genes are most closely related to prognosis in AA and EA will be required in order to focus on the best methylation candidates in Aim 2 and the best antibodies in Aim 3. However, we will not ignore the advantages of additional genome-wide expression and methylation information we continue to obtain from the lower quality samples, which may also allow us to iteratively prioritize certain genes over others for RT-PCR.

4. Impact……..……

- **What was the impact on the development of the principal discipline(s) of the project?**

Extraordinary strides in sensitivity and throughput in nucleic acid quantitation are occurring. These advances may allow us to use old FFPE samples that even with our new approaches, are currently hopelessly degraded and low abundance. We are keeping these samples for when technology allows their use.

- **What was the impact on other disciplines?**

We have observed that the unmethyl-CpG antibody is exquisitely specific to CpG islands, whereas the methyl-CpG antibody assays methylation in a lot of the genome. The difference in the spectrum of capture of the mCpG and CpG antibodies may be exploitable in any system where differential methylation is of interest.

- **What was the impact on technology transfer?**

We are considering filing patents on our results from highly degrades FFPE samples. We are considering protecting the prognosticators. However, we need to process more samples, first.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. Changes/Problems..……

- **Changes in approach and reasons for change**

The tasks and goals remain unchanged. We are adapting to best technical practices.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The quality in the oldest FFPE samples is a problem, but we believe we have learned how to deal with it, as explained above.

- **Changes that had a significant impact on expenditures**

The main technician on the project left *at no notice* in the middle of the year and replacing him took time. We have underspent for the year.

The fall in the cost of Illumina sequencing on the HiSeq 4000 opened an opportunity for low quality samples that would otherwise have been prohibitively expensive. This should be cost neutral by allowing a greater fraction of our huge effort on processing clinical samples to ultimately result in data.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None

- **Significant changes in use or care of human subjects**

None

- **Significant changes in use or care of vertebrate animals.**

Not applicable

- **Significant changes in use of biohazards and/or select agents**

None

6. Products……

- **Publications, conference papers, and presentations**

Rahmatpanah, Farah, Tracy Luu, Pardis Zaeri, Xin Chen, Yuanjie Hu, Joe Adams, Harmony Saunders et al. "Abstract A78: *The role of tumor microenvironment in prostate cancer of African Americans*." *Cancer Epidemiology Biomarkers & Prevention* 24, no. 10 Supplement (2015): A78-A78.

Rahmatpanah, Farah, Zhenyu Jia, Bozhao Men, Parsa Rahmatpanah, Sepideh Madahian, Michael McClelland, and Dan Mercola. "*Methylation correlates with suppressed expression of immunomodulatory genes in the tumor-adjacent stroma of African American Prostate Cancer compared patients of European American ancestry.*" *The FASEB Journal* 30, no. 1 Supplement (2016): 1053-7.

Farah Rahmatpanah, Kathleen McGuire, Michael McClelland, Dan Mercola. *The use of whole genome methylation scanning to define genes preferentially suppressed in African American prostate Cancer*. The Science of Cancer Health Disparities in Racial/ Ethnic Minorities and the Medically Underserved. AACR meeting (Sep 25-28, 2016). Fort Lauderdale, FL. AACR program and proceeding, Abstract # B04, Page 108.

In addition, we were represented by Dr. Lilly at the GU ASCO meeting in January 2016 and the ASCO general meeting in June 2016.

Website(s) or other Internet site(s)

None

Technologies or techniques

In addition to the results of our investigation, some of the innovations, such as in using complementary CpG antibodies, and resurrecting otherwise intractable samples may be of interest as technology manuscripts.

Inventions, patent applications, and/or licenses

Patents on the initial prognosticator were initiated prior to the award of the grant.

Other Products

Sequencing data will be deposited in GEO once it is validated.

7. Participants & Other Collaborating Organizations……

- **What individuals have worked on the project?**

Name:	*Michael McClelland*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-1788-9347
Nearest person month worked:	*2*
Contribution to Project:	*Supervision, data analysis, writing*
Funding Support:	*Effort is 10% of NIH cap. Contributes about 10% more effort from UC Salary.*

Name:	*Dan Mercola*
Project Role:	*Co-PI*
Researcher Identifier (e.g.	0000-0002-0281-9840

ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Supervision, data analysis, writing*
Funding Support:	*Effort is 10% of NIH cap. Contributes about 10% more effort from UC Salary.*

Name:	*Mike Lilly*
Project Role:	*Co-investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Management of AA sample acquisition*
Funding Support:	*Effort beyond the 1% budgeted (MUSC) is donated*

Name:	*Farah Rahmatpanah*
Project Role:	*Assistant Project Scientist*
Researcher Identifier (e.g. ORCID ID):	0000-0002-6158-1692
Nearest person month worked:	*6*
Contribution to Project:	*The primary bench scientist on the project*
Funding Support:	*Effort on this grant*

Name:	*Steffen Porwollik*
Project Role:	*Assistant Project Scientist*
Researcher Identifier (e.g. ORCID ID):	0000-0001-9616-614
Nearest person month worked:	*1*
Contribution to Project:	*Data management*
Funding Support:	*Effort on this grant*

Name:	*Weiping Chu*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Sample processing.*
Funding Support:	*Effort on this grant*

Name:	*Mary Berkaw*
Project Role:	*Technologist*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Sample acquisition and processing*
Funding Support:	*Effort on this grant (MUSC)*

Name:	*Anne Sawyer*
Project Role:	*Staff Research Associate II*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Sample acquisition. Sample logging*
Funding Support:	*Effort on this grant*

Name:	*Rachel Mendelson*
Project Role:	*Junior Specialist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Programming*
Funding Support:	*Effort on this grant*

Name:	*Manuel Sutton*
Project Role:	*Programmer/Analyst II*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Database management*
Funding Support:	*Effort on this grant*

Name:	*Bozhao Men*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Assist with bench work*
Funding Support:	*Left the lab at short notice*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

The following grant is to be awarded.

USDA (McClelland/Andrews) 01/01/17-12/31/20
Definition of Targets to Inhibit Salmonella Colonization of the Intestine
Role: Site Principal Investigator. Effort 1%
Major Goal: Pathways to bacterial colonization of the gut
Overlap: none

The following grant had an extension of one year so it has not expired:

3U01CA086402-14S2 (I Thompson/Mercola). 07/01/14- 06/30/17
"San Antonio Center for Biomarkers of Risk for Prostate Cancer (SABOR)", a multisite study.

The following grants have expired on schedule in the reporting year:

DOD CDMRP PC120465 (Mercola) 09/01/13-08/31/16
Validation of Biomarkers of the Tumor Microenvironment

USDA UF11033 (McClelland/Teplitski) 02/01/11-06/30/16
Salmonella interactions with tomatoes

UC system cancer grant (McClelland) 07/01/15-06/30/16
Exploiting the effect of bacteria on tumors

- **What other organizations were involved as partners?**

  Nothing to report

8. Special Reporting Requirements……

None

9. Appendices……

Meeting abstracts are appended.

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Methylation correlates with suppressed expression of immunomodulatory genes in the tumor-adjacent stroma of African American Prostate Cancer compared patients of European American ancestory

Farah Rahmatpanah[1], Zhenyu Jia[1], Bozhao Men[1], Parsa Rahmatpanah[1], Sepideh Madahian[1], Michael McClelland[1,2] and Dan Mercola[1]

Author Affiliations

[1]Pathology, UCI, Irvine, CA
[2]Microbiology & Molecular Genetics, UCI, Irvine, CA

Abstract

Background The incidence of prostate cancer (PCa) is approximately 60% higher, and the mortality rate is 2 to 3 times greater, among African American men (AA) resident in the U.S. compared with American men with a European background (EA). Men of West African ancestry from the Caribbean and South America share similar incidence and mortality to AAs, suggesting a possible genetic and/or epigenetic contribution to these outcomes.

Studies from our lab have indicated that stroma of tumor-bearing prostate has thousands of significant RNA expression changes compared to normal tissue which correlate with age, grade, and outcome. These important properties of stroma have been exploited to develop biomarker panels (classifiers) from tumor-associated stroma that reliably distinguish normal prostate from tumor-bearing prostate or distinguish good outcome from bad outcome for individual patients. Our studies have identified hundreds of significant differences in gene expression in AA versus EA stroma (1). Remarkably, 92% of expression differences between AA and EA occurred in the stroma component and of these 97% were in the direction of lower expression in AA compared to EA patients. Altered immune/inflammatory processes in tumor-adjacent stroma may be responsible for the aggressive nature of prostate cancer in AA patients (1).

It is possible that differences in DNA methylation in specific genes of immune/inflammatory pathways could contribute, at least in part, to the generally more aggressive behavior of AA compared to EA PCa through suppressing immune response elements.

Methods The methylation and expression status of tumor-adjacent stroma was determined using archived FFPE samples from one AA and two EA prostate cancer cases using ChIP protocols for methylated CpG on a whole genome (NGS) basis. The AA and EA cases were matched for age, time to relapse/survival and Gleason score. Differentially methylated regions in each case were identified using methyl-CpG binding domain of the human MBD2 protein combined with NGS. Transcriptome of tumor stroma was generated using the same exact cases. For the analysis of NGS data (Chip- Seq and RNA-Seq) we used Strand NGS package.

Results Genome-wide methylation analysis revealed 4399 sites of significant ($p < 0.001$) hypermethylation in AA versus EA, and 899 sites of hypermethylation in EA versus AA PCa cases. Pathway analysis of 259 genes that were both methylated in this study and, simultaneously down regulated for RNA expression in tumor stroma of AA compared to EA identified by expression analysis of previous microarray data (1) showed significant enrichment ($p < 0.05$) of immune related pathways such as interleukins (IL) -17, -5, -11, -9 and microRNA targeted genes in leukocytes and lymphocytes, B-cell receptor signaling and interferon type1 response pathways.

Conclusion Our studies provide the first whole gene methylation analysis of PCa FFPE patients and provide insights into the global methylome of the tumor

microenvironment of PCa in two different races. Our preliminary data show that one AA tumor-adjacent stroma has a greater number of hypermethylated regions than in tumor-adjacent stroma of two matched EA patients. The fact that many of the stroma-associated hyper methylated and suppressed genes in AA PCa patients are associated with immune/-inflammatory response pathways implies that epigenetic alterations might be responsible for deficiencies in tumor immunity in AA PCa.

Kinseth et al (2014). Int J Cancer 134, 81-91.

Support or Funding Information

NCI 1 U01 CA152738-01 (PI: Mercola/C. Lee - Co-Pis); 09/01/10-8/31/16. "The Prostate Cancer Tumor Microenvironment Exhibits Differentially Expressed Genes Useful for Diagnosis"

DOD CDMRP (McClelland) 09/15/15-09/14/18Health disparities in African Americans Role: Principal Investigator. Major Goal: Studies of prostate stroma for prognosis.

Footnotes

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Abstract A78: The role of tumor microenvironment in prostate cancer of African Americans

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Abstracts: Seventh AACR Conference on The Science of Health Disparities in Racial/Ethnic Minorities and the Medically Underserved; November 9-12, 2014; San Antonio, TX

Abstract

Background: The incidence of prostate cancer (PCa) is approximately 60% higher and the mortality rate 2 to 3 times greater among African American men (AA) in the U.S. compared to men with a European background, European Americans (EA). Men of West African ancestry from the Caribbean and South America share a similar increase in incidence and mortality to AA suggesting a genetic contribution to these outcomes. We have observed that expression of hundreds of genes in the tumor microenvironment is strikingly different for AA and EA patients1. The majority of significant differences in the stroma of AA patients were down-regulated compared to EA patients. These expression alterations could be related to somatically acquired DNA changes in the stroma but non-tumor tissue in cancer usually exhibits far fewer genetic changes than tumors. Thus, it may be that inherited or distinct environmental changes are responsible for the differences seen between AA and EA stroma. Whatever the basis of the large number of differences observed, the distinct patterns for AA and EA argue strongly, and for the first time, that there are many molecular distinctions between PCa in these races that are resident in the tumor-adjacent microenvironment. We hypothesize that the stroma of AA PCa exhibits a large-scale lower expression of protein mediators of cellular antitumor immunity and reduction in factors involved in cell-cell and cell-extracellular matrix (ECM) contacts. Such differences may, thereby, favor aggressive cancer including early metastases in AA.

Methods: In order to test the basis of the relative decreased expression in AA stroma, ChIP methods for isolating methylated and unmethylated DNA combined with NGS have been used to define methylated sites in two PCa EA cells lines as well as FFPE samples of stroma from EA and AA PCa. To validate the microarray data, we are using Tumor Microarrays from both CA (n=443) and AA (n=105) patients to confirm in immunohistochemistry (IHC) the differential protein expression of many immune and ECM mediators identified in our initial studies.

Results: We validated the ChIP-NGS methods by quantitatively confirming significant methylation within the promoter regions of LnCAP and DU135 cells compared to immortalized normal p69 cells for 35 reported methylated genes including GSTPi. Of the 965 largest down-regulated transcripts observed in AA stroma (1), 237 were observed to exhibit increased promoter methylation in AA stroma tissue compared to EA stroma. These 237 genes identified four main pathways (WNT, TGFβ, integrin mediated cell matrix adhesion, and EMT, all with $p<0.00001$) that are deregulated in AA stroma compared to CA stroma. The IHC studies have shown that CD8+ cell infiltration is significantly greater into the tumors of EA patients than those from AA. Immune mediators of antigen presentation are greater expressed in EA tumors, strongly suggesting immune processes involved in anti-tumor immunity may be more efficient in EA than AA patients. In addition, factors mediating interaction with the ECM such as ITGA5 and ECM formation such as COL4α1 also are increased in EA.

Conclusions: These preliminary studies support the hypothesis that the stroma of AA PCa exhibits a large-scale lower expression of protein mediators of cellular antitumor immunity and reduction in factors involved in cell-cell and cell-ECM contacts. The results suggest a role for the microenvironment in the aggressive features of PCa of AA.

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